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Full Length Research Paper

Genomic diversity among Basmati rice (*Oryza sativa* L) mutants obtained through ^{60}Co gamma radiations using AFLP markers

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Mutation breeding can be considered successful in obtaining new cultivars and broadening the genetic base of rice crop. In order to obtain new varieties of rice with improved agronomic and grain characteristics, gamma radiation (^{60}Co) has been used to generate novel mutants of the Basmati rice. In this study rice cultivars; Basmati-370 and Basmati-Pak, were exposed to different doses of gamma radiations and stable mutants along with parents were studied for genomic diversity on the basis of molecular marker (AFLP). Morphological data showed that mutants of Basmati-370 performed well for yield and yield components and grain physical parameters whereas, the mutant EL-30-2-1 has extra long rain trait as compared to the parent (Basmati-Pak). The genetic variations determined through AFLP revealed a total of 282 scorable bands, out of which 108 (37.81%) were polymorphic. The number of fragments produced by various primers combinations ranged from 11 - 26 with an average of 17.63 fragments per primer combination. Maximum 26 bands were amplified with P-AAG/M-CAG primer combination and minimum one band was amplified with P-ATG/M-CTA primer combination. Two groups of genotypes were detected; group-A had DM-1-30-3-99, DM-1-30-34-99 and EF-1-20-52-04 mutants along with parent Basmati-370, whereas the group-B contained EL-30-2-1 and parent Basmati-Pak. The results of AFLP analysis indicated that the rate of polymorphism was 4.43% (DM-1-30-3-99), 4.25% (DM-1-30-34-99) and 6.38% (EF-1-20-52-04) among the genomes of mutants and parent Basmati-370, respectively, whereas polymorphism rate was 5.32% between genome of EL-30-2-1 and Basmati-Pak. The study further confirmed that the use of gamma radiations is an effective approach for creating new rice germplasm.

Key words: Rice, mutants, (^{60}Co) gamma radiation, AFLP markers.

INTRODUCTION

The presence of rice in food economy of early man has been found around 5000 BC in China and India from archeological excavations (Sharma et al., 2000). In Pakistan, the earliest archeological evidence of rice was obtained from Mohen-jo-daro and Harappa in Sindh and Punjab provinces (Oka, 1988).

Globally, rice (*Oryza sativa* L.) is one of the most important cereal crop and is planted on about one tenth of the earth's arable land. It is the single largest source of food energy to half of humanity (Eckardt, 2000; Kurata and Yamazaki, 2006). In Pakistan, after cotton, rice is 2nd major exportable commodity. It accounts for 5.5% of the total value added in agriculture and 1.1% of the GDP. During 2007 - 2008, area and production figures indicate that rice (aromatic and non-aromatic) was grown on 2.51 million hectares giving production of 5.56 million tonnes

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Table 1. Yield and yield components and grain physical parameters of mutants and parents in yield trial.

Genotype	Origin	Dose (Gy)	Days to 50% flowering	Plant height (cm)	Productive tillers/plant	Panicle fertility (%)	Yield (kg/ha)	Paddy length (mm)	Paddy width (mm)
DM-1-30-3-99	Basmati-370	300	118.3^{ab}	113.5^{e}	19.5^{b}	92.3^{a}	5335^{a}	9.88^{c}	1.88^{e}
DM-1-30-34-99	Basmati-370	300	118.6^{ab}	114.1^{e}	17.3^{bc}	92.1^{a}	5160^{b}	9.40^{d}	2.15^{a}
EF-1-20-52-04	Basmati-370	200	103.6^{d}	126.9^{d}	16.6^{bc}	94.7^{a}	5440^{a}	9.41^{d}	1.98^{c}
EL-30-2-1	Basmati-Pak	300	117.3^{b}	143.9^{c}	24.3^{a}	74.6^{b}	3743^{e}	13.37^{a}	2.07^{b}
Basmati-370	Parent		115.3^{c}	166.2^{a}	15.0^{c}	93.6^{a}	3932^{d}	8.73^{e}	1.92^{d}
Basmati-Pak	Parent		120.0^{a}	152.7^{b}	17.5^{bc}	96.1^{a}	4057^{c}	10.08^{b}	1.96^{cd}

with average yield of 2211 kg per hectare (Anonymous 2007 - 2008a). The contribution of Basmati rice in total production was 2.64 million tonnes from an area of 1.46 million hectares with an average yield of 1801 kg per hectare (Anonymous 2007-2008b). In future, the prospects to expand area for cultivation of rice will be limited due to increased competition for land and water from urban and industrial sector in major rice producing countries of Asia (Kueneman, 2006).

To enhance the grain yield and quality with the limited resources are the two major objectives of Basmati rice breeding program. Rice grain size (grain length and breadth) and shape (length/breadth ratio) are important traits of rice breeding (Luo et al., 2004) and have a direct effect on commercial success of improved rice cultivars. In most Asian countries, commercial cultivars belong to the medium or long grain class. Long and slender grained Basmati cultivars of India and Pakistan command premium prices in the international market (Redona and Mackill, 1998).

In China, substantial increase in rice yield (15 - 20%) has been achieved through hybrid varieties (Riveros, 2000) but hybrid breeding of aromatic Basmati rice of Pakistan is linked with few problems viz; 1) narrow base of genetic material, 2) crossing with elite indica lines does not produce full spectrum of recombinants through hybridization, 3) scarcity of donor parents for grain quality and 4) the fact that Basmati rice is poor combiner (Saleem et al., 2005; Saleem, 2009). It is, therefore, necessary to concentrate efforts on widening the genetic base and exploration of best combiners to produce superior genotypes without loss of traditional Basmati quality. Conventional techniques have not been able to resolve these problems. So, broadening the genetic base of rice is an essential pre-requisite for rice improvement programme. Among different approaches, induced mutation can be used for the development of Basmati rice varieties/germplasm with good agronomic and grain characteristics (Rashid et al., 2003; Alvarez et al., 2000).

The estimation of genetic diversity on the basis of morphological traits alone does not determine actual level of diversity among germplasm because morphological traits are the product of gene and environmental interactions (Alan, 2007). Therefore, selection based merely on morphological traits has been often misleading (Kumar et al., 1998; Bibi et al., 2009). During last decade, techniques based on DNA markers along with morphological traits have been used to detect variation at DNA level to distinguish closely related genotypes. The development of the Amplified Fragment Length Polymorphism (AFLP) (Vos et al., 1995) allowed genome characterization in several crops (Zhu et al., 1998; Prashanth et al., 2002; Witkowicz et al., 2003; Malone et al., 2006). The specific objective of this study was to use the AFLP technique for the evaluation of genomic diversity among the advanced rice mutants with their parents obtained through gamma radiations. The information, thus obtained, will be helpful to develop an effective rice-breeding programme.

MATERIALS AND METHODS

Plant materials

Pure, dry and dormant seeds with moisture content of 12% of Basmati cultivars cvs; Basmati-370 and Basmati-Pak were irradiated with different doses of gamma rays (150, 200, 250 and 300 Gy). M_1 generation was planted in the same season at NIAB, Faisalabad, Pakistan. One seedling/hill spaced 10 cm apart between and within rows was grown in the field. Main panicle from each M_1 plant was harvested at physiological maturity and was bulked dose wise for growing M_2 population next year. From M_2 population desirable mutants were isolated on the basis of early flowering, short stature, good plant type and grain characteristics. The homozygosity of the selected mutants was evaluated for in M_3 and M_4 progeny rows along with untreated controls.

Four mutants and their respective parents originated from different doses of gamma rays (Table 1) used in yield trial were triplicated at NIAB, Faisalabad, Pakistan. Thirty days old seedlings were transplanted into a 1.50 x 2.50 (sq. m.) plot size with one seedling/hill. Standard agronomic practices and plant protection measures were adopted. The data were collected on seven characters viz; days to 50% flowering, plant height, productive tillers/plant, panicle fertility, yield, paddy length and paddy width. Observations were recorded on 10 randomly selected plants of the middle rows excluding the border plants. Their means were compared according to Duncan's Multiple Range (DMR) test (Steel and Torrie, 1980).

DNA extraction

Genomic DNA was isolated from fresh green leaves of 20 day-old seedlings of four mutants and their parents. Each sample comprising

Table 2. Details of primers used in AFLP analysis.

Pre-selective primers for amplification				
P*st*-I specific primer core sequence +A: M*se*-I specific primer core sequence +C:			P*st*-A 3' M*se*-C 3'	
Selective amplification primer over-hangs				
P*st*I specific M*se*I specific	P*st*-AAG M*se*-CAC	P*st*-ATG M*se*-CTA	P*st*-AAC M*se*-CAG	P*st*-ATC M*se*-CTG
Eight primer combinations used				
	P*st*-AAG + M*se*-CAC	P*st*-AAG + M*se*-CTA	P*st*-AAG + M*se*-CAG	P*st*-AAG + M*se*-CTG
	P*st*-ATG + M*se*-CAC	P*st*-ATG + M*se*-CTA	P*st*-ATG + M*se*-CAG	P*st*-ATG + M*se*-CTG
	P*st*-AAC + M*se*-CAC	P*st*-AAC + M*se*-CTA	P*st*-AAC + M*se*-CAG	P*st*-AAC + M*se*-CTG
	P*st*-ATC + M*se*-CAC	P*st*-ATC + M*se*-CTA	P*st*-ATC + M*se*-CAG	P*st*-ATC + M*se*-CTG

of 400 mg of ground, lyophilized tissues was processed using CTAB method as per Saghai- Maroof et al. (1984) with minor modifications until the crude DNA extracted. The crude DNA was then washed twice with 75% ethanol absolute taking care that the DNA pellet should not be disturbed, dissolved in 50 ul TE buffer (10 m*M*Tris (pH = 8.0), 1 m*M*EDTA (pH = 8.0) and treated with RNAase-A. Tubes were caped and racked over night at room temperature to dissolve DNA. The quality of the extracted DNA was evaluated by electrophoresis on 0.8% agarose. The purity of the genomic DNA was estimated by the ratio of absorbance at 260 and 280 nm (A^{260}/A^{280}) by using Cintra 10-e machine.

AFLP analysis

The AFLP method of Vos et al. (1995) was performed using the AFLP Analysis System I Kit (GIBCO BRL, Life Technologies) with minor modifications. Genomic DNA was double-digested with P*st*I/M*se*I enzyme combinations and the digested DNA fragments were ligated with P*st*I and M*se*I adapters. The pre-amplification step was carried out with AFLP primers having one selective nucleotide (P*st*I+A, M*se*I+C) and the PCR products were diluted in a ratio of 1:20 with Tris-EDTA buffer (pH = 8.0) and then used as template for the selective amplification. Selective amplification was performed with three selective nucleotides (P*st*I+ANN, M*se*I+CNN). Sixteen primer combinations (P-AAG / M-CAC, P-AAG / M-CTA, P-AAG / M-CAG, P-AAG / M-CTG, P-ATG / M-CAC, P-ATG / M-CTA, P-ATG / M-CAG, P-ATG / M-CTG, P-AAC / M-CAC, P-AAC / M-CTA, P-AAC / M-CAG, P-AAC / M-CTG, P-ATC / M-CAC, P-ATC / M-CTA, P-ATC / M-CAG, P-ATC / M-CTG) were tested (Table 2). PCR was amplified in the PTC-100, Pettier Thermal Cycler, M. J. Research Inc., following cycles; 94°C for 30 s, 65°C for 30 s, 72°C for 1 min., (-0.7°C/cycle) during 12 cycles until the optimal annealing temperature of 56°C reached, resulting in a total of 23 cycles which were necessary for complete amplification. Formamide loading buffer (80 ul) was added to the PCR products. The samples were loaded on a 6.0% polyacrilamide gel under standard sequencing conditions. AFLP fingerprints were visualized using the silver nitrate staining method according to the manufacturer's instruction (Promega). Each primer combination was scored by eye for the number and sharpness of polymorphic fragments detected. The scored fragments ranged in size from 200 to 700 base pairs (bp). The size of the fragments was determined by comparing sequencing ladders of control template DNA to AFLP patterns.

Only polymorphic and clearly repeatable bands between 200 and 700 bp were used for analysis. Gels were scored visually for the presence (1) or absence (0) of bands. Associations among lines were determined from cluster analysis based on genetic distance estimates. The unweighted pair group method with arithmetic averages (UPGMA) was used for hierarchical clustering using appropriate procedures of the computer package NTSYS-PC version 2.10e.

RESULTS AND DISCUSSION

Performance in yield trial

Data on yield and yield components and grain physical parameters of the mutants along with their parents were recorded (Table 1) and it was noted that early flowering mutant (EF-1-20-52-04) matured 12 days earlier than the parent (Basmati-370) which is desirable for wheat-rice cropping pattern. Plant height reduced significantly (28.91 %) in both the dwarf mutants, that is, DM-1-30-3-99 and DM-1-30-34-99 as compared to the parent (Basmati-370). Reduction in plant height prevents lodging under heavy fertilization and strong winds. The mutants DM-1-30-3-99, DM-1-30-34-99 and EF-1-20-52-04 had greater agricultural yield than Parent Basmati-370. The yield contributing traits also improved in the mutants as compared to their parents. In case of paddy yield, mutants of Basmati-370 gave 35.7% (DM-1-30-3-99), 31.2% (DM-1-30-34-99) and 38.4% (EF-1-20-52-04) higher paddy yield as compared to the parent respectively. The mutant developed from Basmati-Pak at 300Gy was poor in yield due to low panicle fertility % (74.6%) but it has extra long grain length, that is, 13.37 mm as compared to parent (10.08 mm) (Table 1 and Figure 5). This mutant can be used in hybrid breeding programme to improve the grain physical parameters of Basmati rice. Gonzalez et al. (2008) reported the similar results on rice by using protons radiations. It is also reviewed that various physical and chemical mutagens are known to act in different ways to cause DNA lesions (Micke et al., 1990; Chopra, 2005). Morphological comparison of the mutants with parents is shown in Figures 3 and 4 respectively.

Our knowledge on genetic diversity and relationships of advanced rice genotypes in Basmati rice needs to be strengthened. However the data from this study showed that morphological characters alone still cannot be regarded

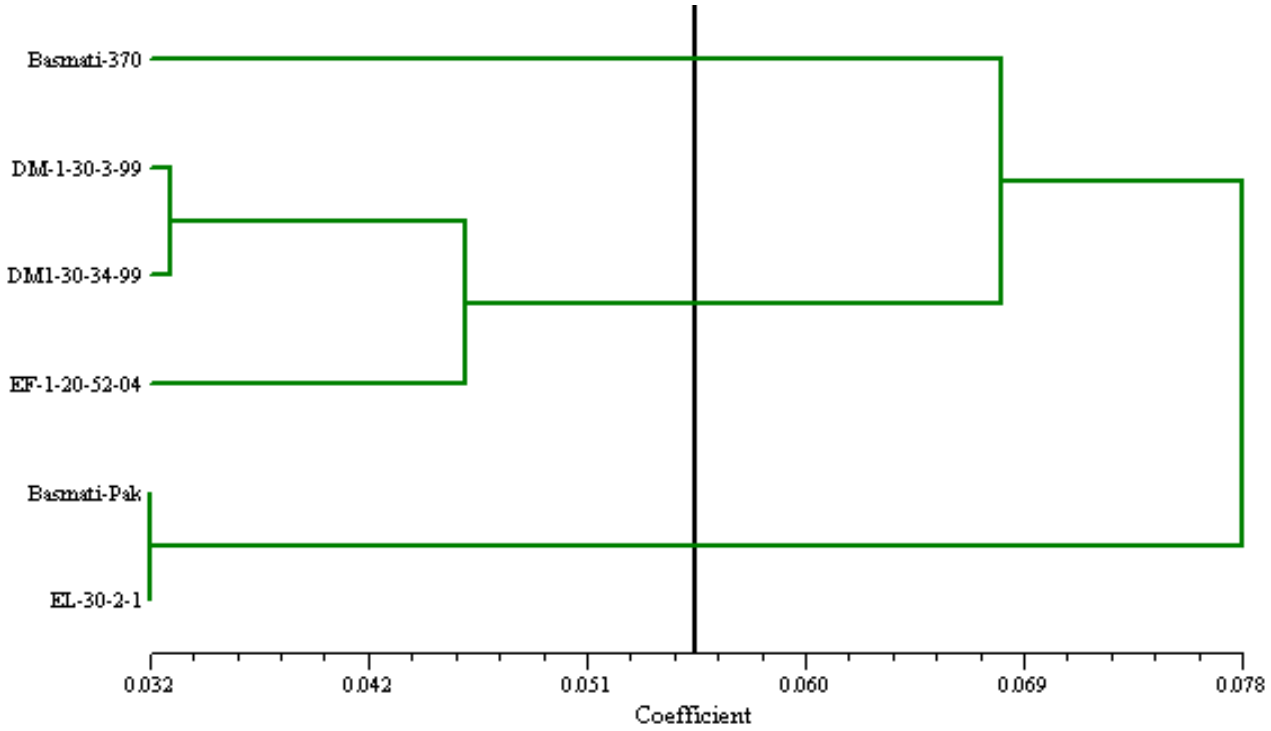


Figure 1. Dendrogram of the six rice genotypes developed from AFLP data using unweighted pair group method of arithmetic means (UPGMA).

as critical indicators to identify individual rice genotype because of the environmental influence and nutrition available to the plant from the soil where it grows. Compared with the morphological characters, the AFLP is more powerful technique to study the relation-ships and variability among rice germplasm and the re-solution is much higher to identify individual varieties, even to those with the same label (Alan, 2007).

Molecular evaluation

AFLP analysis effectively detects large numbers of polymorphic genetic loci in a single PCR reaction (Figure 2). The number of scored bands and polymorphic bands obtained for the different primer combinations used are illustrated show in Table 3. The sixteen primer combinations tested produced good polymorphism results showing a total of 282 bands with fragments sizes ranged from approximately 200 to 700 bp. Of these, 108 bands were clearly polymorphic between the genotypes exhibitting 37.81% of amplified polymorphic fragments per primer combination with a range of 1 - 26 (8.33 to 75.00% of polymorphism) as shown in Table 3. On an average 17.63 bands were per primer pair were produced.

Analyses of the genetic distance between lines based on the Dice index allowed us to define 2 groups. The dendrogram in Figure 1 depicted the clustering of rice mutants into two groups of individuals as well as a clear distinction between mutants and the parents. Group-A is composed by three mutants (DM-1-30-3-99, DM-1-30-34-99 and EF-1-20-52-04); whilst EL-30-2-1 mutant was present in group-B, showing a very small genetic distance between them. The mutant EF-1-20-52-04 more genetically distant from all mutants.

Compared with the AFLP products, the identical bands in the mutants viz; DM-1-30-3-99 (257), DM-1-30-34-99 (258) and EF-1-20-52-04 (246) were recorded when compared with the parent Basmati-370 whereas EL-30-2-1 had 246 identical bands with parent Basmati-Pak. The mutants of Basmati-370 were exhibiting polymorphism rate as 4.43% (DM-1-30-3-99), 4.25% (DM-1-30-34-99) and 6.38% (EF-1-20-52-04) respectively whereas the mutant EL-30-2-1 revealed 5.32% polymorphism rate between parent Basmati-Pak. Similar results were reported by Xing et al., 2004.

The level of variation detected using AFLP techniques depends on the combination of restriction enzymes selected for the first stage, on the number of combinations of primers used and on the genetic distance between the genotypes analyzed. The AFLP technique permits the detection of the variation of many loci simultaneously. AFLP markers are widely used for the evaluation of genetic variation between genotypes with a differentiated degree of relatedness, especially between genotypes with a small genetic distance. In this case, the detection of a polymorphism requires the use of a sensitive marker system, due to an unusual low frequency of

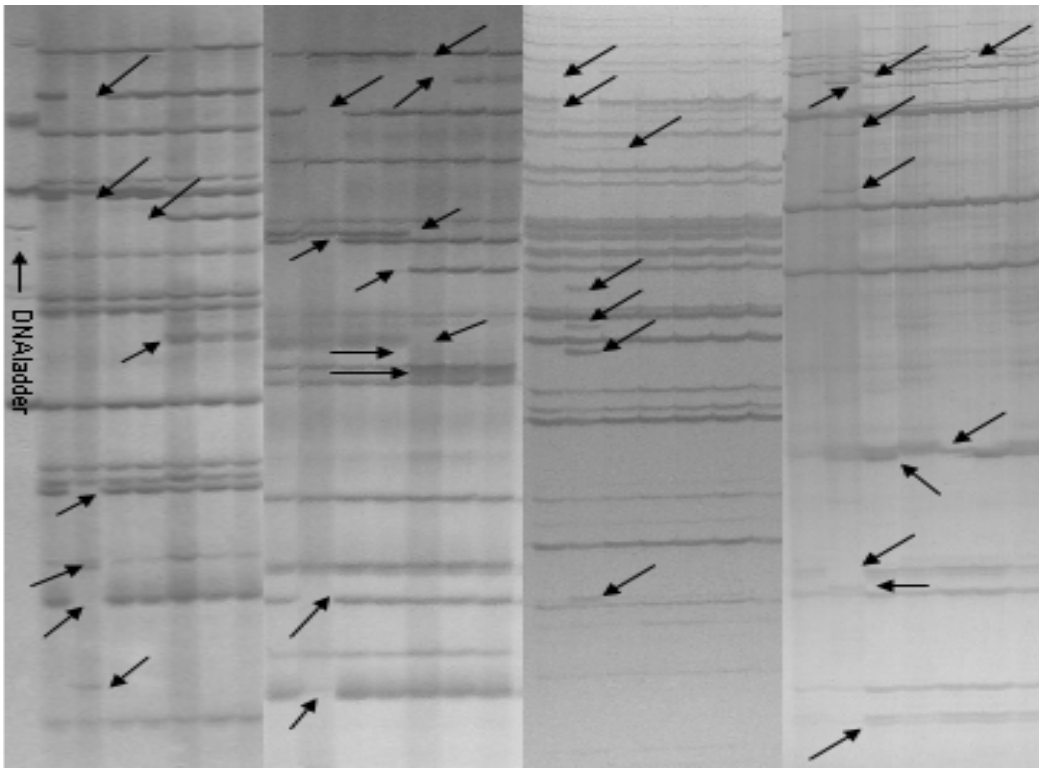


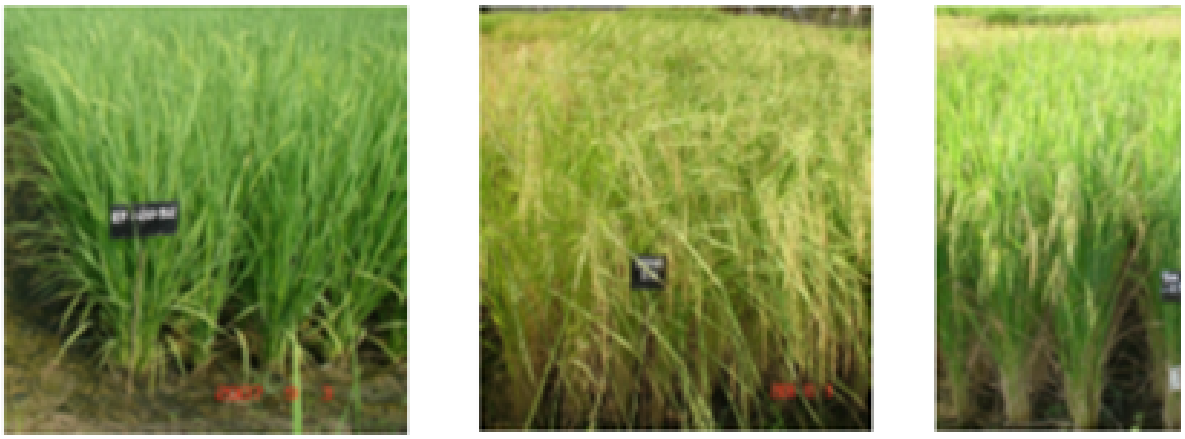
Figure 2. AFLP band pattern amplified by primer combination P-ATC/M-CTA, P-AAC/M-CAG, P-AAG/M-CAG and P-ATG/M-CAG from left to right, respectively.

Table 3. Primer combinations with polymorphism (%) obtained through AFLP technique.

S/N	Primer combination	Bands scored	Polymorphic bands	Polymorphism (%)
1	P-AAG / M-CAC	17	10	58.82
2	P-AAG / M-CTA	17	7	41.18
3	P-AAG / M-CAG	26	7	26.92
4	P-AAG / M-CTG	21	8	38.09
5	P-ATG / M-CAC	11	2	18.18
6	P-ATG / M-CTA	12	1	08.33
7	P-ATG / M-CAG	21	10	47.62
8	P-ATG / M-CTG	13	7	53.85
9	P-AAC / M-CAC	15	4	26.67
10	P-AAC / M-CTA	22	10	45.45
11	P-AAC / M-CAG	25	11	44.00
12	P-AAC / M-CTG	17	3	17.65
13	P-ATC / M-CAC	13	3	23.07
14	P-ATC / M-CTA	21	8	38.09
15	P-ATC / M-CAG	19	8	42.11
16	P-ATC / M-CTG	12	9	75.00
	Total	282	108	37.81

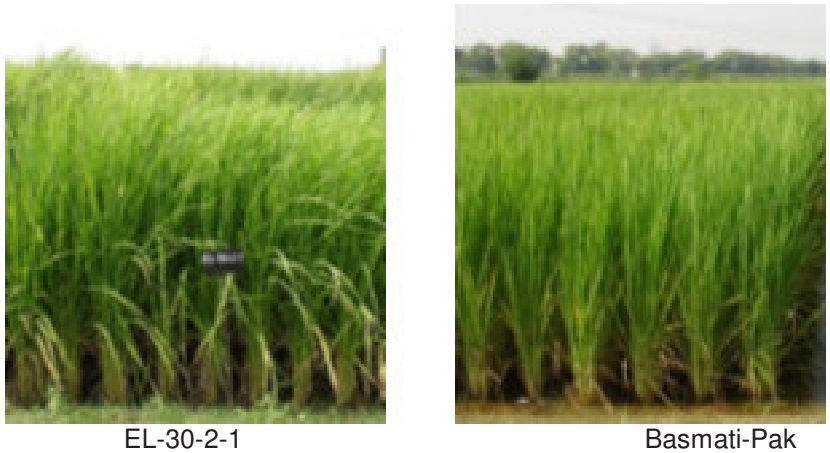
mutations (Witkowicz et al., 2003).

The present work concluded the efficiency of AFLP markers to identify rice mutants for desirable agronomic and quality parameters previously difficult in conventional means. The findings would definitely be utilized for marker assisted breeding programme to widen the genetic base and to develop Basmati rice cultivars with typical quality of Basmati rice.



DM-1-30-34-99 EF-1-20-52-04
Basmati-370

Figure 3. Morphological comparison of DM-1-30-34-99, EF-1-20-52-04 with parent Basmati-370.



EL-30-2-1 Basmati-Pak

Figure 4. Morphological comparison of EL-30-2-1 mutant with parent Basmati-Pak.

Figure 5. Cooking and physical grain comparison of EL-30-2-1 mutant with parent Basmati-Pak.

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